Final synopsis of the thesis entitled

ANTIDIABETIC EFFECT OF *POUTERIA CAMPECHIANA* (KUNTH) BAEHNI IN STREPTOZOTOCIN INDUCED DIABETIC SPRAGUE-DAWLEY RATS

by

Akshata Pai

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Prof. Chandrakala Shenoy K.

Department of Biosciences
Mangalore University
Mangalagangothri-574199
Karnataka

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INTRODUCTION AND REVIEW OF LITERATURE

Diabetes mellitus (DM) is one of the most common and serious chronic metabolic disorders characterised by a high blood sugar level over a prolonged period resulting from defects in insulin secretion, insulin action, or both (Ramachandran *et al.,* 2011). DM can be classified into clinical categories as Type 1 diabetes mellitus and Type 2 diabetes mellitus. Type 1 diabetes (T1DM) is caused by an autoimmune reaction where the body's immune system attacks the insulin-producing beta cells in the islets of the pancreas gland. As a result, the body produces none to very little insulin with a relative or absolute deficiency of insulin (International Diabetes Federation (IDF), 2021). In type 2 diabetes (T2DM), hyperglycaemia is the result of inadequate production of insulin and the inability of the body to respond fully to insulin, defined as insulin resistance (Galicia-Garcia *et al.,* 2020). During a state of insulin resistance, insulin is ineffective and therefore initially prompts an increase in insulin production to reduce rising glucose levels but over time a state of relatively inadequate production of insulin can develop (International Diabetes Federation, 2017). Chronic hyperglycaemia causes serious long-term complications including cardiovascular disease, stroke, chronic kidney disease, damage to nerves, damage to the eyes and cognitive impairment (American Diabetes Association, 2009).

India has the second-largest number of diabetics worldwide. According to an estimate, over 74 million Indians were diagnosed with diabetes in 2021, and this is expected to rise to over 124 million by 2045. Type 2 diabetes accounts for over 90 percent of all diabetic cases in India (Kanwal, 2023). The worldwide population of diabetic patients is increasing alarmingly with India claiming the number one position with 50.8 million diabetics; a prediction made by the WHO stated that developing countries would face the impact of the epidemic in the 21st century; The population was predicted to come under this syndrome by 2010 was 285 million people (6.4% of world's adult population) and expected to exceed to 438 million by 2030 (7.8% of the world's adult population) (Paul and Paul, 2012).

According to IDF, 537 million people are living with diabetes and predicted to reach 643 million by 2030 and 783 million by 2045. Prevalence has been rising more rapidly (3 in 4 adults) in low- and middle-income countries over the past few decades. Diabetes is responsible for 6.7 million deaths in 2021 (International Diabetes Federation, 2021).

Plant-based drugs have been used worldwide in traditional medicines for the treatment of various diseases (Muthukrishnan and Sivakumar, 2018). Medicinal plant parts

are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and linins. These compounds can be used for therapeutic purposes or as precursors for pharmaceutical synthesis due to their antioxidant activity (Patil, 2020). As a result, global market demand for herbs and their products has increased tremendously in recent years, especially in developing countries (Ekor, 2014).

World Health Organization (WHO) is now more solicitude with the safety and efficacy of the plant-derived herbal drug during usage. Natural antioxidants are present in almost all plants that can maintain beta cell function and prevent diabetes-induced reactive oxygen species formation (Ujwala *et al.,* 2015). Many compounds isolated from plant sources have been reported to show antidiabetic activity (Rahman *et al.,* 2022). Diabetes mellitus can directly involve serum lipid levels that cause diabetic dyslipidaemia (Belayneh and Birru, 2018). Liver enzyme markers are also associated with insulin resistance and type 2 diabetes mellitus (Noroozi Karimabad *et al.,* 2022).

Medicinal plants have been used to treat various ailments for decades (Parsaeimehr *et al.,* 2017). The genus Pouteria belongs to the family Sapotaceae which is widely found around the world (Silva *et al.,* 2009). *Pouteria campechiana* (Kunth) Baehni is commonly known as canistel, yellow sapote, or egg fruit. *P. campechiana* is an evergreen tree and 6 -12m tall with slender and glossy leaves (Chan Zapata *et al.,* 2017). The bark has been used to treat fevers and skin eruptions, while the seeds have been used to treat ulcers (Aly *et al.,* 2016). The fruit of *P. campechiana* is reported as a rich source of carotenoids (Agostini-Costa *et al.,* 2010) and is well-known as an antioxidant and hepatoprotective (Aseervatham *et al.,* 2014). The ethyl acetate fraction of *P. campechiana* leaves possesses anti-mitotic activity (Hernandez *et al.,* 2008).

However, there are fewer scientific reports in the modern literature regarding its usefulness in treating diseases. Thus, the present investigation was focused on the antidiabetic effect of *Pouteria campechiana* extract on diabetic rats.

SCOPE OF THE STUDY

Diabetes is recognized as one of the leading causes of morbidity and mortality in the world. The current treatment for the control of Diabetes Mellitus includes diet, exercise, oral antidiabetic drugs, and insulin therapy. However, insulin and other oral hypoglycaemic drugs show adverse effects. Due to this adverse effect, nowadays Herbal Drugs have great demand in the treatment of Diabetes. Drugs from plants have been known

to be safe and cheaper. The bioactive compound present in plants exhibits anti-diabetic activity more effectively than the oral hypoglycaemic agents used in clinical therapy. These actions are supposed to be exhibited due to the cumulative effect of phenols, glycosides, terpenoids, and flavonoids present in the plant extracts. Hence, the present study is focused on a medicinal plant, *Pouteria campechiana* which is used in folk medicine. *In vitro* and *in vivo* rat models are used to evaluate the antidiabetic activity of phytoconstituents present in plant extract that record significant antioxidant activity.

OBJECTIVES

- To evaluate the physicochemical properties and phytochemicals of *Pouteria campechiana* (Kunth) Baehni leaf and fruit.
- To assess the safety profile of methanolic extract from the leaf and fruit of *P. campechiana* (Kunth) Baehni.
- To evaluate the *in-vitro* and *in-vivo* antidiabetic activity of *P. campechiana* (Kunth) Baehni leaf and fruit methanolic extract.
- To identify the bioactive constituents by GC-MS analysis and to elucidate the mechanisms underlying the antidiabetic activity of *P. campechiana* (Kunth) Baehni leaf and fruit methanolic extract by molecular docking.

GENERAL MATERIALS AND METHODS

Collection of plant material:

The leaves and fruits of *P. campechiana* (Kunth) Baehni were collected from Bellikoth, Kasargod District, Kerala. The plant material was identified and authenticated in the Department of Botany, Kerala University, Kariavattom. The voucher specimens were preserved in the botany department herbarium library (accession no: KUBH 10170).

The fresh leaves and fruits of *P. campechiana* were collected and shade dried for about 5 weeks and 20 weeks respectively, at an ambient temperature of 30°C. Dried leaf and fruit materials were then subjected to pulverization to get coarse powder, which was stored at room temperature for further studies.

Preparation of plant extract:

The *P. campechiana* leaf and fruit extraction was carried out by the Hot continuous Soxhlet extraction using 70% methanol and concentrated by rotary evaporator and then dried and stored in airtight bottles in a refrigerator at 4°C for further use. The percentage extractive yields of the methanolic extract of leaves and fruits were recorded.

Experimental animals:

Healthy young adult Sprague-Dawley rats (SD) weighing 180-200 g, aged 8-10 weeks were used for the study. All rats were housed in stainless steel, open-mesh cages in a room maintained under controlled conditions of 20 – 22°C, a 12-hour light-dark cycle, and 40-60% relative humidity with optimal air changes per hour as per CPCSEA guidelines. The animals were fed ad libitum with a commercial rat maintenance diet from AMRUT laboratory animal feed manufactured by Pranav agro-industries Limited, Sangli.

Induction of Diabetes:

The streptozotocin - Nicotinamide model was used to induce Type 2 Diabetes mellitus. Diabetes was induced using Streptozotocin (STZ) prepared in 50mM Citrate buffer and administered single-dose STZ at 45mg/kg b.wt. (i.p.) Nicotinamide (NAD) was administered 15 min before STZ injection at 230mg/kg b.wt. (i.p.).

Statistical analysis:

All the results were expressed as the mean ± standard deviation (SD) or mean ± standard error mean (SEM). Physicochemical and phytochemical parameters were evaluated by student t-test. Statistical comparisons were made between the treated groups and the control in acute oral toxicity and the treated group and diseased control in antidiabetic activity using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests or a two-way ANOVA followed by Bonferroni's multiple comparison test. Values $p < 0.05$ were considered statistically significant. The data was computed in GraphPad Prism 5.

BRIEF OUTLINE OF THE STUDY

The thesis is organized into four chapters:

Chapter 1: Physico-chemical and phytochemical screening of *Pouteria campechiana* leaf and fruit.

- Physicochemical, fluorescence, and EDX analysis of *P. campechiana* leaf and fruit powder.
- Phytochemical analysis of a methanolic extract of *P. campechiana* leaf and fruit.

Chapter 2: *In-vitro* and *in-vivo* safety evaluation of *Pouteria campechiana* leaf and fruit methanolic extract.

- Cytotoxicity of *P. campechiana* leaf and fruit extract.
- Acute oral toxicity of *P. campechiana* leaf and fruit extract.

Chapter 3: *In-vitro* and *in-vivo* antidiabetic activity of *Pouteria campechiana* leaf and fruit methanolic extract.

- *In vitro* antioxidant activity of *P. campechiana* leaf and fruit extract.
- *In vitro* anti-diabetic activity of *P. campechiana* leaf and fruit extract.
- Evaluation of anti-hyperglycemic action of *P. campechiana* extracts in Streptozotocin (STZ) induced diabetic rats.
- Evaluation of the anti-hyperlipidemic activity of *P. campechiana* extracts in STZ-induced diabetic rats.
- Evaluation of the anti-atherogenic activity of *P. campechiana* extracts in STZ-induced diabetic rats.
- Effect of *P. campechiana* extract on liver enzymes and kidney functions test.
- *In vivo* antioxidant effect of *P. campechiana* extract in STZ-induced diabetic rats.
- Evaluation of the restoration of the altered architecture of liver, pancreas and kidney in STZ-induced diabetic rat using *P. campechiana* extract.

Chapter 4: Identification of bioactive constituents by GC-MS analysis and elucidation of mechanism underlying the antidiabetic activity of *P. campechiana* by molecular docking.

- Identification of bioactive constituents of *P. campechiana* leaf and fruit methanolic extract using GC-MS analysis.
- Molecular docking between phytochemicals which serve as ligands and selected proteins which serve as receptors that play an essential role in different metabolic pathways in diabetes.

Chapter 1: Physico-chemical and phytochemical screening of *Pouteria campechiana* leaf and fruit.

1.1 Evaluation of the physicochemical, fluorescence, and EDX analysis of P. campechiana leaf and fruit powder.

Since *P. campechiana* has been used in traditional medicine to treat various ailments, it is a prerequisite to standardize the drug for its use. Physicochemical parameters are important for detecting adulteration or improper handling of drugs (Nagani *et al.*, 2011). Physicochemical standardization of the *P. campechiana* leaves and fruit powder was performed as per WHO guidelines and procedures listed in the Indian pharmacopoeia. Fluorescence analysis of the *P. campechiana* leaf and fruit powder was carried out according to the methods of Chase and Pratt (1949). *P. campechiana* leaf and fruit powder were subjected to elemental analysis using a scanning electron microscope (SEM) with an energy-dispersive X-ray spectrometer (EDX).

From this study, the results of different types of ash values may provide a basis for identifying the purity and quality of the drug. The lesser amount of the three variables of ash values (Total ash, acid-insoluble ash, and water-soluble ash) indicates that the inorganic matter and silica were less in *P. campechiana* leaf and fruit powder which suggests that the powder is not adulterated. The low moisture content of leaf and fruit helps in the reduction of the growth of bacteria, yeast, or fungi through storage. Assessment of the nature of powder can be identified by extractive value and also helps to assist in the evaluation of the solubility of specific constituents in a particular solvent (Sudha and Srinivasan, 2013). In the present study, the percentage extractive value of leaf and fruit was higher in water than in alcohol, which signifies that a large number of phytoconstituents of leaf and fruit were more soluble in water than alcohol.

Fluorescence analysis is a rapid method for the resolution of a doubtful specimen. When physical and chemical methods are insufficient, the plant material may be identified from its adulterants based on fluorescence characteristics (Chumbhale and Upasani, 2012). Many phytocompounds do not fluoresce in daylight, but under ultraviolet light they emit fluorescence. If these components are not fluorescent by nature, other reagents can typically be used to transform them into fluorescent derivatives (Jeevitha *et al.*, 2021). As a result, several crude drugs are frequently evaluated qualitatively, which is an essential pharmacognostic indicator (Chase and Pratt, 1949). In treatment with various chemical reagents under UV and visible light, both the leaf and fruit powder of *P. campechiana* displayed distinctive colouration.

The SEM-EDX spectra and elemental compositions were also analysed. Leaf powder showed the presence of various elements such as N, O, Cl, K, Ca, and C, in which O was in the highest percentage followed by C while small quantities of N, Cl, K, and Ca were also detected. Similarly, N, O, K and C were detected in *P. campechiana* fruit powder. O was found in the highest percentage followed by C while a small amount of N and K were also present in the fruit powder of *P. campechiana*.

1.2 Evaluation of the phytochemical analysis of P. campechiana leaf and fruit methanolic extract.

Phytochemical analysis of plants detects the presence of various phytoconstituents which are known to reveal medicinal use in addition to the action on the human body (Rakholiya *et al.*, 2016). The presence of these secondary metabolites indicates that the plant might be of medicinal significance. The leaf and fruit extracts of *P. campechiana* prepared were analysed for the preliminary phytochemical screening as per standard protocols. The tests were detected by visual observation of colour change or by precipitate formation. Based on preliminary phytochemical screening, quantitative phytochemical analysis such as estimation of total phenolic content (TPC), total flavonoid content (Jose and Anilkumar, 2018), alkaloids (Roghini and Vijayalakshmi, 2018), and total tannin content (Ci and Indira, 2016) were analysed according to the standard procedure.

Total phenol contents, flavonoid content and tannin content were found high in *P. campechiana* leaf extract (PCL) than in fruit extract (PCF). Total alkaloid was found to be higher in PCF than in PCL. The qualitative and quantitative phytochemical investigation provide beneficial information and ideas about the different phytoconstituents which reveals the medicinal use of *P. campechiana*.

Chapter 2: *In-vitro* and *in-vivo* safety evaluation of *Pouteria campechiana* leaf and fruit methanolic extract.

2.1 In vitro cytotoxicity evaluation of P. campechiana leaf and fruit extract

Toxicity testing can reveal some of the risks that may be associated with the use of medicinal plants, especially in sensitive populations. Cytotoxicity is generally used to predict potential toxicity using cultured cells that may be normal or transformed cells (Kundishora *et al.*, 2020).

The murine 3T3-L1 cell line was procured from the National Centre for Cell Science (NCCS), Pune. The Cell viability of 3T3-L1 cells was determined using a 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) assay. The methanolic extracts obtained from *P. campechiana* leaf and fruit were tested for cytotoxicity assay. PCL and PCF had no significant cytotoxic activity when compared with the normal control.

2.2 In vivo acute oral toxicity evaluation of P. campechiana fruit and leaf extract

An acute toxicity test is the first step to determine the unfavourable effects of substances within 14 days of administration of a single dose via oral routes (Alelign *et al.*, 2020). All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) of Skanda Life Sciences (Organisation), Bidadi, Bangalore, Karnataka, India under proposal no. IAEC-SLS-2021-037. Acute oral toxicity was performed according to the Organisation for Economic Cooperation and Development (OECD) 423. The experiments were performed using healthy young adult female Sprague-Dawley rats (SD).

50, 300, and 2000 mg/kg b.wt of *P. campechiana* leaf and fruit methanolic extract were used to study toxicity. After dosing, all the rats were observed daily to record any symptoms of ill-health or behavioural changes for 14 days. On day 15, all rats were anaesthetized and blood samples were collected for the estimation of haematological (Erba Transasia; H360) and biochemical parameters (Erba Chem 5X). Following blood collection, the animals were humanely sacrificed and subjected to gross necropsy examination, and vital organs such as the liver, kidney, heart, and lung were collected and preserved in 10% formalin for histopathological examination.

In this toxicity study, oral administration of a single dose of *P. campechiana* leaf and fruit methanolic extracts ranging from 50mg/kg to 2000mg/kg caused no signs of toxicity or mortality in any of the animals studied during the observational period of 14 days. There were no changes in haematological and biochemical parameters as well as histological

architecture of vital organs. Accordingly, the LD_{50} is greater than 2000mg/kg. Based on the Globally Harmonized System of Classification and Labelling of Chemicals, substances having an LD_{50} value greater than 2000mg/kg are considered relatively safe (Ebbo *et al.,* 2020; Muñoz *et al.,* 2021). The results obtained from the acute toxicity study showed that the PCF and PCL were revealed to be safe at 2000mg/kg of body weight.

Chapter 3: *In-vitro* and *in-vivo* antidiabetic activity of *Pouteria campechiana* leaf and fruit methanolic extract.

3.1 In vitro antioxidant activity of *P. campechiana* leaf and fruit extract.

P. campechiana leaf and fruit methanolic extract were analysed for *in vitro* antioxidant activity. A modified thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenate as a lipid-rich medium (Annappa and Rudrappa, 2013). DPPH assay scavenging activity was assessed based on the antioxidant activity of the methanolic extracts and their scavenging activity of the stable free radical 1, 1- diphenyl2-picryl hydrazyl (DPPH) (Pant *et al.,* 2015). Total antioxidant capacity (TAC) was determined using a phosphomolybdenum reagent (Basumatary *et al.,* 2015). Reducing power assay was estimated for measuring the ferrous ion chelation ability (Ngonda, 2013).

Egg yolk lipids undergo rapid nonenzymatic peroxidation when hatched in the presence of ferrous sulphate (Khan *et al.,* 2012). The PCL and PCF extract exhibited lipid peroxidation inhibition in a concentration-dependent manner. The highest activity was observed in PCL than in PCF. In the DPPH assay, an antioxidant present in plant extract causes discolouration of the molecule from violet to yellow. DPPH IC_{50} value of PCL is less compared to fruit, which shows that leaves possess more antioxidant activity compared to fruit. Phosphomolybdenum assay is based on the reduction of Mo_{VI} into Mo_{V} by the compounds present in the plant extract with the formation of a green-blue phosphate-Mo_{V} complex (Bhatti *et al.,* 2015). The antioxidant capacity of PCL and PCF extract shows in a concentration-dependent manner. The ferric-reducing antioxidant power assay evaluates the reducing potency of the antioxidant in a plant extract. This antioxidant donates a hydrogen atom to a ferric complex and produces a blue-coloured compound called ferrous (Nagarajan *et al.,* 2017). The higher the antioxidant concentration, the higher the amount of ferrous (Fe^{2+}) which shows evidence of high absorbance of the test sample. PCL showed higher chelating activity than PCF.

3.2 In vitro anti-diabetic activity of *P. campechiana* leaf and fruit extract.

The major enzymes involved in the absorption of glucose from the gut include the pancreatic α-amylase and α-glucosidase enzymes (Kajaria *et al.,* 2013). One of the approaches to tackling DM has been the use of agents that inhibit the action of enzymes involved in the absorption and metabolism of carbohydrates. Inhibition of these enzymes regulates the postprandial blood sugar level in Type 2 DM patients by reducing the absorption of sugars from the gut. The assay for α-amylase inhibition was performed as described by Kumar *et al.,* (2011). The α-glucosidase inhibitory activity was measured as described by Dayananda and Reddy (2015). A glucose uptake assay was carried out by using the 2-(N-(7-nitrobenz-2-oxa1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) flow cytometric method in 3T3-L1 cell lines. 2-NBDG is a fluorescent glucose analogue which represents the amount of glucose uptake by cells. Measuring the amount of fluorescence by a flow cytometric method in cells stained with 2- NBDG gives an accurate estimation of the glucose uptake by the cells.

In the present study, the crude extract of PCL and PCF showed α-amylase and α-glucosidase inhibitory activities in a dose-dependent manner. The mechanism by which the crude extracts of PCL and PCF exert their inhibitory effects could be attributed to the presence of a high content of polyphenols, predominantly flavonoids. Plant polyphenols have been shown to inhibit both α-amylase and α-glucosidase activities (Da Costa Mousinho *et al.,* 2013).

Adipocyte differentiation inhibitors may be effective in preventing various diseased conditions such as obesity, atherosclerosis, diabetes, and other associated complications. Under the appropriate conditions, 3T3-L1 cells are known to differentiate into adipocytes and are used as a model for adipose cells, which are one of the major sites of lipid and glucose metabolism (Prathapan *et al.,* 2012). The decrease in glucose uptake in type 2 diabetes is due to the insulin resistance developed by the cells. A fluorescent deoxyglucose analogue, 2-NBDG is used as a probe for the detection of glucose taken up by cultured 3T3-L1 cells (Dubey *et al.,* 2018). In this study, experiments on the cellular uptake of glucose revealed that *P. campechiana* can induce glucose utilization in 3T3-L1 cells. 2-NBDG uptake was observed more in higher concentrations of leaf and fruit extract when compared to lower concentrations. An increase in fluorescence intensity corresponds to an enhanced uptake of 2-NBDG into the cells, which indicates that a compound (PCL and PCF) possesses insulin-mimetic properties and implies that they have excellent therapeutic and potential anti-diabetic properties.

3.3 Evaluation of anti-hyperglycemic action of P. campechiana extract in Streptozotocin (STZ) induced diabetic rats.

The study aims to determine the antidiabetic activity of methanolic extracts of *P. campechiana* (Kunth) Baehni in Sprague-Dawley rats (SD rats). All animal experiments were approved by the IAEC of Skanda Life Sciences (Organisation), Bidadi, Bangalore, Karnataka, India under proposal no. IAEC-SLS-2020-003 for antidiabetic study. All rats were maintained as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines. The experiments were performed using healthy young adult male Sprague-Dawley rats (SD).

Experimental Design and Treatment:

The treatment groups (n=6) were as follows:

Group I: Normal control - Rats treated with vehicle (0.5% CMC) (2ml/kg b.wt p.o.)
Group II: Diseased control - Rats treated with streptozotocin (STZ) (45mg/kg b.wt i.p.)
Group III: Positive control - Rats treated with STZ + glibenclamide (5mg/kg b.wt p.o.)
Group IV: Rats treated with STZ + *P. campechiana* fruit extract (250mg/kg b.wt p.o.)
Group V: Rats treated with STZ + *P. campechiana* fruit extract (500mg/kg b.wt p.o.)
Group VI: Rats treated with STZ + *P. campechiana* leaf extract (250mg/kg b.wt p.o.)
Group VII: Rats treated with STZ + *P. campechiana* leaf extract (500mg/kg b.wt p.o.)

Fasting blood glucose (FBG) was measured 2 weeks after STZ injection and animals with 200 to 300mg/dL FBG were considered for the study. Animals were treated daily with respective test samples and glibenclamide for 14 days. FBG was measured on 0^{th}, 3^{rd}, 5^{th}, 7^{th}, and 14^{th} day. On day 15, rats were anaesthetized with ketamine and blood samples were collected for the estimation of haematological (Erba Transasia; H360) and biochemical parameters (Erba Chem 5X). Following blood collection, the animals were humanely sacrificed and subjected to gross necropsy. The kidney, liver, and pancreas were collected and preserved in 10% formalin for histopathological examination to analyse the effect of *P. campechiana* fruit (PCF) and *P. campechiana* leaf (PCL) extract on different organs of the rats. The pancreatic homogenate was used to estimate the tissue antioxidants.

The normal control group was found to be stable in their body weight but a significant reduction in the body weight was observed in the STZ-induced diabetic rats. The decrease in weight in diabetics is due to the continuous excretion of glucose and a decrease in the peripheral uptake of glucose and glycogen (Sivagnanam *et al.,* 2022). Treatment with

PCL and PCF extracts and the standard glibenclamide shows a statistically significant increase in body weight when compared with the diseased control.

Experimental studies on day 14 revealed that the PCF and PCL extract produced a significant decrease in the fasting blood glucose level (FBG) when a comparison was done with the diabetic control group. The possible mechanism by which PCL and PCF brought about its hypoglycemic action in diabetic rats might be by potentiating the insulin effect of plasma, by increasing either the pancreatic secretion of insulin from the existing beta cells or by its release from the bound form (Patel *et al.,* 2012).

In diabetes mellitus, the haemoglobin (Hb) level is decreased, because the surplus glucose produced in the body reacts with the haemoglobin to form glycosylated haemoglobin (Devaki *et al.,* 2016). Diseased control showed decreased Hb level and increased glycosylated haemoglobin (HbA1C) level, upon treatment with the PCL 500 mg/kg and glibenclamide, the Hb levels and HbA1C level were brought back to normal control which could be due to an improvement in insulin secretion from remnant pancreatic beta cells in diabetic rats.

Streptozotocin reduces the synthesis of mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), which indicates a defect in Hb synthesis and a defect in osmotic pressure control and osmolality of the plasma hence the number of red blood cells (RBC), Hb, MCH, MCHC, and Packed cell volume (PCV) decreased significantly in diabetic control rats (Fazelipour *et al.,* 2021). Plant extract may contain some phytochemicals that can stimulate the formation or secretion of erythropoietin in the stem cells of the rats. This hormone enhances rapid synthesis of RBC which is supported by the improved level of MCH, and MCHC (Oyedemi *et al.,* 2011). Administration of PCL and PCF extract could improve the level of PCV, MCH and MCHC. The antihyperglycemic effects resulting from treatment with plants are usually assigned to their ability to improve the activity of pancreatic tissue, by increasing insulin secretions or by reducing the intestinal absorption of glucose (Salehi *et al.,* 2019). Similarly, administration of methanolic extracts of PCL and PCF for 14 days significantly reduced plasma glucose concentration and increased plasma insulin level in STZ-induced diabetic rats. PCL (500mg/kg) has shown a comparable activity with glibenclamide-treated groups which is a standard antidiabetic drug that stimulates insulin secretion from beta cells of islets of Langerhans. Therefore, the present study result indicates the insulin-stimulating property of the extract.

3.4 Evaluation of the anti-hyperlipidemic activity of P. campechiana extract in STZ-induced diabetic rats.

Hyperlipidaemia was characterized most often by increased triglyceride and reduced HDL cholesterol levels. This is generally observed in both type 1 and type 2 diabetes, representing the defect of insulin action in each, either due to inadequate secretion or resistance. It is well known that hyperlipidaemia is accepted as an independent risk factor for cardiovascular disorders (CVD) in diabetic patients (Sabitha *et al.*, 2011). Diabetes induced by STZ in rats significantly elevated the Total cholesterol (TC), Triglycerides (TG), Low-Density Lipoprotein cholesterol (LDL), and Very Low-Density Lipoprotein cholesterol (VLDL) levels and decreased the High-Density Lipoprotein cholesterol (HDL) levels compared with normal control rats. In the present study, administration of PCF and PCL to diabetic rats showed a significant reduction in TG, LDL, and VLDL levels and increased HDL levels when compared with the diseased rats. These actions of PCL and PCF directly support its ability to reduce hyperlipidemia in diabetes and hence, it may prevent CVD related to diabetes.

3.5 Evaluation of the anti-atherogenic activity of P. campechiana extract in STZ-induced diabetic rats.

The atherogenic index and the coronary risk index were very high in diabetic rats. Standard drug and plant extract significantly reduced the atherogenic indices (AIP), and coronary risk index (CRI) when compared to diabetic rats. PCF and PCL exhibited potent antiatherogenic activity by reducing the atherogenic coefficient. Percentage protection was increased with the dose, PCL has shown more protection than PCF.

3.6 Effect of P. campechiana extract on liver enzymes and kidney functions

Enzymes indicating liver damage, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels increased in diabetic rats. The elevated serum level of these enzymes was significantly reduced by PCL 500 mg/kg treatment. There is evidence that diabetic complications such as increased gluconeogenesis and ketogenesis may be due to elevated enzymes. The restoration of transaminases to their normal levels after treatment indicates a revival of insulin secretion and regenerative activities of islets of Langerhans cells of the pancreas after administration of the plant extract (Toma *et al.,* 2015). The present result indicates the ameliorative effect of the extract on the

hepatocytes of the liver after the action of the injurious agent (STZ) in the diabetic state (Asante *et al.,* 2016).

Increased levels of blood urea nitrogen (BUN) and serum creatinine (sCr) in diseased control indicates prolonged hyperglycemia which causes damage to the nephrons of the kidney. Nephrons are damaged due to high blood sugar levels (Biri *et al.,* 2022). PCF (500 mg/kg) and PCL (250 mg/kg and 500 mg/kg) improved renal functions in diabetic rats by reducing BUN and PCL 500 mg/kg reduce sCr levels in diabetic rats. Therefore, the results demonstrate that PCL can normalize vital organ function in rats more than PCF. A decrease in total bilirubin in diabetic rats as observed in the present study may be a result of its endogenous antioxidant activity. Bilirubin decreases the levels of free oxygen radicals, thus, ameliorating oxidative stress and causing worse diabetic regulation (E *et al.,* 2018). Treatment with PCL 500mg/kg extract was able to reverse this condition in diabetic rats to normalcy. Low serum albumin concentrations suggest chronic damage to the liver as a result of infection (Sunmonu and Afolayan, 2013). The restoration of total protein and albumin concentrations in the serum of STZ-diabetic rats by glibenclamide, PCL and PCF extract of both doses extracts may be due to the inhibition of proteolytic activity due to enhanced insulin secretion and proper utilization of blood glucose (Sundaram *et al.,* 2009).

3.7 In vivo antioxidant effect of P. campechiana extract in STZ-induced diabetic rats.

P. campechiana exhibits efficient *in vitro* antioxidant activity as described in 3.1. However, it needs to be detected whether this ROS scavenging ability is also applicable *in vivo*. Thus, the assays for endogenous antioxidants (Superoxide dismutase (SOD), Catalase (CAT), Glutathione s-transferase (GST) and Glutathione peroxidase (GPx)) in pancreatic tissue were carried out. The activity of SOD and GPx was lowered in diabetic rats, probably due to glycation of the enzyme due to hyperglycaemia (Singh *et al.,* 2013). The pancreas of diabetic rats showed a significant decrease in SOD, CAT, GST, and GPx as compared with the normal control. The polyphenols, flavonoids and various other antioxidants present in PCL and PCF can scavenge ROS thereby protecting the endogenous antioxidants hence, treatment of diabetic rats with PCL and PCF showed a significant increase in SOD, CAT, GST, and GPx in the pancreas.

3.8 Evaluation of the restoration of the altered architecture of liver, pancreas and kidney in STZ-induced diabetic rat using P. campechiana extract.

In the present study, the biochemical findings observed in diabetic rats conform with histopathological alterations of β cells of the pancreas and hepatocytes. Such histopathological alterations were reduced by the administration of PCL and PCF extract at both doses. Histopathology of the kidney of STZ-induced diabetic rats showed degeneration of glomeruli, tubular tissue damage and mononuclear cell infiltration. STZ-induced diabetic rat treatment with PCL and PCF extract improves the injured rat kidney with increasing doses of treatment. Liver histology of STZ-induced diabetic rats showed loss of normal architecture of the liver like hepatocellular injury and dilation of the central vein (CV). Treatment with different doses of PCL and PCF extract improved the histological condition. Histopathology of the pancreas of STZ-induced diabetic rats displayed a severe degenerative change of pancreatic islets and extensive necrosis. STZ-induced diabetic rats treated with PCL and PCF extract and glibenclamide restored the necrotic changes and normal cellular population.

Chapter 4: Identification of bioactive constituents by GC-MS analysis and elucidation of mechanism underlying the antidiabetic activity of *P. campechiana* by molecular docking.

4.1 Evaluation of bioactive constituents of P. campechiana leaf and fruit methanolic extract using GC-MS analysis.

The chemical constituents of *P. campechiana* were characterized by GC-MS analysis using a GC-MS model (QP 2010 Plus, Shimadzu). Through GC-MS analysis, phytoconstituents present in plants can be identified and that gives a clear picture of the pharmaceutical value of the plant (Selvi and Basker, 2012). The relative percentage of constituents present in the methanolic extract was expressed as a percentage with peak area normalization. The bioactive compounds present in the methanolic extract were identified by comparing the retention time and patterns of the mass peak using the Wiley Registry of Mass Spectral Data, New York (Wiley8) and the database of the National Institute Standard and Technology 11 (NIST 11).

The GC-MS chromatogram shows the presence of nine compounds in the methanolic extract of the *P. campechiana* leaf and twelve compounds in the methanolic extract of the *P. campechiana* fruit by comparing their retention times and interpreting the mass spectra of the compound. The results obtained were identified based on a comparison

of their mass spectra with those of NIST 11 and Wiley Libraries. The compounds identified in the crude methanolic extract of the leaf are; N-(2-Cyano-Ethyl)-N-Methyl- Acetamide, Chinasaure, calendin, Aspidocarpine, Octadecanoic acid methyl ester, Dibutyl phthalate, Kaur-16-ene, 11,14,17- eicosatrienoic acid methyl ester, Phytol acetate. The compounds identified in the crude methanolic extract of fruit are; Acetic acid pentyl ester, Dimethylamine, N-(Neopentyloxy)-, Glycerin, 4H- Pyran-4-one, 2,3-Dihydro-3,5-Dihydroxy-6-methyl-, 2-pentene, 3-ethyl-4,4-Dimethyl-, 5-keto-2,2-dimethylheptanimine, N, N- dimethyl-O- (1-methyl-butyl) hydroxylamine, DL-Arabinitol, β – D-Glucopyranoside methyl, 3-Deoxy-d-mannoic lactone, d-Glycero-d-tallo-heptose, Stigmast-5-EN-3-OL,(3 β).

4.2 Molecular docking between phytochemicals which serve as ligands and selected proteins which serve as receptors that play an essential role in different metabolic pathways in diabetes.

Molecular docking analysis was performed to evaluate the most preferred geometry of the protein-ligand complex. The three-dimensional (3D) X-ray crystallographic structures of 6 proteins were retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (http://www.pdb.org) and saved in PDB format. Proteins with PDB ID: 1IR3-Insulin receptor, 2F70-Protein tyrosine phosphatase 1-beta (Kaushik *et al.,* 2014), 1LWO-Glycogen phosphorylase (Herowati and Widodo, 2014), 2G63-Dipeptidyl peptidase IV, 2PRG-Peroxisome proliferator-activated receptor (Verma *et al.,* 2021), 1V4S-Glucokinase (Rathinavel *et al.,* 2019) were used as targeted diabetic receptor proteins. The 3D structures of bioactive compounds from methanolic *P. campechiana* leaf and fruit extract through GC-MS analysis were collected from PubChem, a compound database. The docking studies were carried out for phyto-ligands using the AutoDock program to elucidate the binding affinities to the target proteins.

AutoDock Vina is a majorly used computational docking program in binding studies (Ahmed *et al.,* 2017). After the docking process, the ligands were classified according to their protein-ligands binding energies. The results were analysed based on the interactions between protein and the compounds as well as the binding energies of the complexes. Target protein and chemical compounds are the requirements to start the molecular docking procedure. Binding energy (kcal/mol) of selected phytochemical compounds with the different proteins was done using AutoDock Vina. Kaur-16-ene and aspidocarpine had a binding affinity similar to glibenclamide with some of the receptors on average.

CONCLUSION

Most of the species of the Sapotaceae family are considered to be rich in vitamins and nutritive values and bear medicinal importance, used in ayurvedic preparation. Hence the present study aimed to reconnoitre the *Pouteria campechiana* (Kunth) Baehni leaf and fruit for its protective effect against streptozotocin-induced diabetes in rats. Leaf and fruit powder samples of *P. campechiana* showed the preferable range of all parameters (physicochemical and fluorescence) indicating the purity, efficacy, and safety of both samples for further study. EDS analysis also showed the presence of some elements that play an important role in maintaining normal glucose tolerance such as potassium and calcium.

The methanolic extract of *P. campechiana* leaf and fruit revealed no cytotoxicity against 3T3-L1 cells, which concludes that these extracts could be recommended as a nutraceutical in treating Diabetes Mellitus. The acute toxicity study of methanolic extract of *P. campechiana* leaf and fruit was carried out according to OECD guideline 423 and the results showed no mortality and without notable signs of toxicity up to the dose of 2,000 mg/kg body weight. So, the extracts are safe for long-term administration.

P. campechiana leaf and fruit crude extracts possess significant antioxidant activity and protect the cells against free radical damage. *P. campechiana* leaf and fruit extract showed an inhibitory effect on α-amylase and α-glucosidase enzymes. The experiments on glucose uptake assay by flow cytometry in the 3T3-L1 cell line showed promising results when compared to that of the standard drug metformin. The results of the present study indicated that methanolic extract of *P. campechiana* leaf and fruit extract reduced the glucose level and elevated plasma insulin in streptozotocin-induced diabetic rats. The treatment of rats with *P. campechiana* leaf and fruit extract for 14 days was effective in controlling hyperglycaemia, atherogenicity, hyperlipidaemia, and oxidative damage.

Methanolic extract of *P. campechiana* leaf and fruit were found to be rich in phytoconstituents such as phenols, flavonoids, tannin, and alkaloids which play a major role in the pharmacological activity and were identified by GC-MS analysis. The compounds detected through GC-MS are used for molecular docking studies and based on the docking score, the docking of ligands was done using certain receptors. Kaur-16-ene had a binding affinity similar to glibenclamide with some of the receptors on average.

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PAPER PUBLICATION

- Pai, A., & Shenoy, C.K. (2020). Physicochemical, phytochemical, and GC–MS analysis of leaf and fruit of *Pouteria campechiana* (Kunth) Baehni. *Journal of Applied Biology & Biotechnology*, 8(4): 90-97. ISSN / EISSN: 2455-7005 / 2347-212X; Cited by 6; https://www.scopus.com/sourceid/21100970232

- Pai, A., & Shenoy, C.K. (2021). *In-vitro* antidiabetic activity of methanolic extract of leaves and fruits of *Pouteria campechiana* (Kunth) Baehni. *Biomedicine.* 41(4): 756-762. https://www.scopus.com/sourceid/28618

PAPER PRESENTATION

- Presented a research paper on Phytochemical analysis and evaluation of antioxidant activities of *Pouteria campechiana* (Kunth) Baehni leaves at the International Conference on Contriving Therapeutic Drugs from Medicinal Plants (CTDMP-2020) at the Department of Biochemistry, Sree Narayana Guru College, Coimbatore on12th February 2020.

- Presented a research paper on the Evaluation of antioxidant activity and acute toxicity of *Pouteria campechiana* (Kunth) Baehni leaves extract at International Conference on Advanced Biology 2022 at Inter-University Centre for Evolutionary and Integrative

Biology, University of Kerala, Kariavattom, Thiruvananthapuram on 23rd to 25th February 2022.

- Presented a research paper on Anti-hyperlipidemic activity and anti-atherogenic activity of *Pouteria campechiana* fruit methanolic extract in streptozotocin-induced diabetic rats in the International Conference on Recent Advances in Biological Sciences 2023 organised by Inter-University Centre for Evolutionary and Integrate Biology, University of Kerala, Kariavattom, Thiruvananthapuram on 17th to 19th January 2023.

- Presented a research paper on Phytochemical analysis and anti-hyperlipidemic activity of *Pouteria campechiana* leaf methanolic extract in STZ-induced diabetic rats in the International Conference cum Workshop on Plant Molecular Biology and Bioinformatics jointly organised by the Department of Bioinformatics, Pondicherry University, Indian Science Congress Association (ISCA), Pondicherry Chapter and Society for Plant Research (SPR), Pondicherry on 13th to 15th February 2023.

Mangalagangothri Research Scholar
Date:

Mangalagangothri Research Guide
Date: